Application for United States Letters Patent

To all whom it may concern:

Be it known that we, Thomas M. Jessell, James Briscoe, Johan Ericson, John L.R. Rubenstein, and Maike Sander

have invented certain new and useful improvements in

GENETIC DEMONSTRATION OF REQUIREMENT FOR NKX6.1 AND NKX2.2 IN VENTRAL NEURON GENERATION

of which the following is a full, clear and exact description.

62166/JPW/EMW

GENETIC DEMONSTRATION OF REQUIREMENT FOR NKX6.1 AND NKX2.2 IN VENTRAL GENERATION

This application is a continuation-in-part of U.S. Serial No. 09/569,259, filed May 11, 2000, the contents of which are hereby incorporated by reference into the present application.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

BACKGROUND OF THE INVENTION

During the development of the embryonic central nervous system (CNS) the mechanisms that specify regional identity and neuronal fate are intimately linked (Anderson et al. 1997; Lumsden and Krumlauf 1996; Rubenstein et al. 1998). In the ventral half of the CNS, for example, the secreted factor Sonic hedgehog (Shh) has a fundamental role in controlling both regional pattern and neuronal fate (Tanabe and Jessell 1996; Ericson et al. 19976; Hammerschmidth et al. 1997). Shh appears to function as a gradient signal. In the spinal cord, five distinct classes of neurons can be generated in vitro in response to two- to threefold changes in the

concentration of Shh, and the position at which each neuronal class is generated in vivo is predicted by the concentration required for their induction in vivo (Ericson et al. 1997a; Briscoe et al. 2000). Thus, neurons generated in more ventral regions of the neural tube require progressively higher concentrations of Shh for their induction.

The genetic programs activated in neural progenitor cells in response to Shh signaling, however, remain incompletely defined. Emerging evidence suggests that homeobox genes function as critical intermediaries in the neural response to Shh signals (Lumsden and Krumlauf 1996; Tanabe and Jessell 1996; Ericson et al. 1997; Hammerschmidt et al. 1997; Rubenstein et al. 1998). Several homeobox genes are expressed by ventral progenitor cells, and their expression is regulated by Shh. Gain-of-function studies on homeobox gene action in the chick neural tube have provided evidence that homeodomain proteins are critical for the interpretation of graded Shh signaling and that they function to delineate progenitor domains and control neuronal subtype identity (Briscoe et al. 2000). Consistent with these findings, the pattern of generation of neuronal subtypes in the basal telencephalon and in the ventral-most region of the spinal cord is perturbed in mice carrying mutations in certain Shh-regulated homeobox genes (Ericson et al. 1997; Sussel et al. 1999; Pierani et al., unpublished).

Members of the *Nkx* class of homeobox genes are expressed

by progenitor cells along the entire rostro-caudal axis of the ventral neural tube, and their expression is dependent on Shh signaling (Rubenstein and Beachy 1998). Mutation in the *Nkx2.1* or *Nkx2.2* genes leads to defects in ventral neural pattering (Briscoe et al. 1999; Sussel et al. 1999), raising the possibility that *Nkx* genes play a key role in the control of ventral pattering in the ventral region of the CNS. Genetic studies to assess the role of *Nkx* genes have, however, focused on only the most ventral region of the neural tube. A recently identified *Nkx* gene, *Nkx6.1*, is expressed more widely by most progenitor cells within the ventral neural tube (Pabst et al. 1998; Qiu et al. 1998; Briscoe et al. 1999), suggesting that it may have a prominent role in ventral neural patterning. Here experiments show that in mouse embryos *Nkx6.1* is expressed by ventral progenitors that give rise to motor (MN), V2, and V3 neurons. Mice carrying a null mutation of *Nkx6.1* exhibit a ventral-to-dorsal switch in the identity of progenitor cells and a corresponding switch in the identity of the neuronal subtype that emerges from the ventral neural tube. The generation of MN and V2 neurons is markedly reduced, and there is a ventral expansion in the generation of a more dorsal V1 neuronal subtype. Together, these findings indicate that *Nkx6.1* has a critical role in the specification of MN and V2 neuron subtype identity and, more generally, that *Nkx* genes play a role in the interpretation of graded Shh signaling.

SUMMARY OF THE INVENTION

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

This invention also provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises: a) obtaining a nucleic acid sample from the subject; b) sequencing the nucleic acid sample; and c) comparing the nucleic acid sequence of step (b) with a Nkx6.1 nucleic acid sequence from a subject without motor neuron degenerative disease, wherein a difference in the nucleic acid sequence of step (b) from the Nkx6.1 nucleic acid sequence from the subject without motor neuron degenerative disease indicates that the subject has the motor neuron degenerative disease.

This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises: a) obtaining a nucleic acid sample from the subject; b) performing a restriction digest of the nucleic acid sample with a panel of restriction enzymes; c) separating the resulting nucleic acid fragments by size fractionation; d) hybridizing the resulting separated nucleic acid fragments with a nucleic acid probe(s) of at least 15 nucleotide capable of specifically hybridizing with a unique sequence included within the sequence of a

nucleic acid molecule encoding a human Nkx6.1 protein, wherein the sequence of the nucleic acid probe is labeled with a detectable marker, and hybridization of the nucleic acid probe(s) with the separated nucleic acid fragments results in labeled probe-fragment bands; e) detecting labeled probe-fragment bands, wherein the labeled probe-fragment bands have a band pattern specific to the nucleic acid of the subject; and f) comparing the band pattern of the detected labeled probe-fragment bands of step (d) with a previously determined control sample, wherein the control sample has a unique band pattern specific to the nucleic acid of a subject having the motor neuron degenerative disease, wherein identity of the band pattern of the detected labeled probe-fragment bands of step (d) to the control sample indicates that the subject has the motor neuron degenerative disease.

This invention provides a method of treating neuronal degeneration in a subject which comprises implanting in diseased neural tissue of the subject a neural stem cell which comprises an isolated nucleic acid molecule which is capable of expressing homeodomain Nkx6.1 protein under conditions such that the stem cell is converted into a motor neuron after implantation, thereby treating neuronal degeneration in the subject.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1U **Selective changes in homeobox gene expression in ventral progenitor cells in *Nkx6.1* mutant embryos.** (Figs. 1A-1C) Expression of *Nkx6.1* in transverse sections of the ventral neural tube of mouse embryos E9.5. (Fig. 1A) Expression of *Nkx6.1* is prominent in ventral progenitor cells and persists in some post-mitotic motor neurons at both caudal hindbrain, E10.5, (Fig. 1B) and spinal cord, E12.5, (Fig. 1C) levels. (Fig. 1D, and 1E) Summary diagrams showing domains of homeobox gene expression in wild-type mouse embryos (Fig. 1D) and the change in pattern of expression of these genes in *Nkx6.1* mutants (Fig. 1E), based on analyses at E10.0 - E12.5. (Figs. 1F-1I) Comparison of the domains of expression of *Nkx6.1* (Figs. 1F, 1J) *Dbx2* (Figs. 1G, 1H, 1K, 1L) and *Gsh1* (Figs. 1I, 1M) in the caudal neural tube of wild-type (Figs. 1F-1I) and *Nkx6.1* mutant (Figs. 1J-1H) embryos. (Fig. 1J) Horizontal lines, approximate position of dorsoventral boundary of the neural tube; vertical lines, expression of *Dbx2* and *Gsh1*. Expression of Sonic hedgehog, *Shh* (Figs. 1N, 1R), *Pax7* (Figs. 1N, 1R), *Nkx2.2* (Figs. 1O, 1S), *Pax6* (Figs. 1P, 1S), *Dbx1* (Figs. 1P, 1T) and *Nkx2.9* (Figs. 1Q, 1U)

in wild-type (Figs. 1N-1Q) or *Nkx6.1* mutant (Figs. 1R-1U) embryos at spinal (Figs 1N-1P, 1R-1T) and caudal hindbrain levels (Figs 1Q, 1U). Arrowheads, approximate position of the dorsal limit of *Nkx6.1* expression. Scale bar shown in J= 100μm (Figs. 1A-1C); 50μm (Figs. 1F-1M) or 60μm (Figs. 1N-1U).

Figure 2A-2T. Disruption of motor neuron differentiation in *Nkx6.1* mutant embryos.

The relationship between the domain of *Nkx6.1* expression (Figs. 2A-2C, green) by ventral progenitors and the position of generation of motor neurons and V2 interneurons (Figs. 2A-2D) in the ventral spinal cord of E10.5 wild-type embryos. (Fig. 2A) Isl1/2 motor neurons; (Fig. 2B) HB9 motor neurons; (Fig. 2C) *Lhx3* (Lim3) expression (red) by motor neurons, V2 interneurons and their progenitors is confined to the *Nkx6.1* progenitor domain. (Fig. 2D) *Chx10* (green) V2 interneurons coexpress *Lhx3* (red). Expression of *Isl1/2* (Figs. 2E, 2I), *HB9* (Figs. 2F, 2J), *Lhx3* (Figs. 2G, 2K) and *Phox2a/b* (Figs. 2H, 2L) in the ventral spinal cord (Figs. 2E, 2F, 2G) and caudal hindbrain (Fig. 2H) of E10.5 wild-type (Figs. 2E-2H) and *Nkx6.1* mutant (Figs. 2I-2L) embryos. Pattern of expression of *Isl1/2* and *Lhx3*

at cervical (Figs. 2M, 2N, 2Q, 2R) and thoracic (Figs. 2O, 2P, 2S, 2T) levels of E12.5 wild-type (Figs. 2M-2P) and Nkx6.1 mutant (Figs. 2Q-2T) embryos. Arrows, position of *Isl1* dorsal D2 interneurons. (Figs. 10Q-10T) Absence, position of *Isl1/2* dorsal D2 interneurons. Scale bar shown in I = 60μm (Figs. 2A-2D); 80μm (Figs. 2E-2L); 120μm (Figs. 2M-2T).

Figures 3A-3J **Motor neuron subtype differentiation in *Nkx6.1* mutant mice.**

Depletion of both median motor column (MMC) and lateral motor column (LMC) neurons in *Nkx6.1* mutant mice. Expression of *Isl1/2* (red) and *Lxh3* (green) in E12.5 wilt-type (Figs. 3A, 3C) and *Nkx6.1* mutant (Figs. 3B,3D) mice spinal cord at forelimb levels (Figs. 3E-3J). Motor neuron generation at caudal hindbrain level (Figs. 3E, 3F) *Nkx6.1* expression in progenitor cells and visceral motor neurons in the caudal hindbrain (rhombomere [r] 7/8) of E10.5-E11 wild-type (Fig. 3E) *Nkx6.1* mutant (Fig. 3F) mice. *HB9* expression in hypoglossal motor neurons in E10.5-E11 wild-type mice (Fig. 3G) and *Nkx6.1* mutant (Fig. 3H) mice. Coexpression of *Isl1* (green) and *Phox2a/b* (red) in wild-type (Fig. 3I) or *Nkx6.1* mutant (Fig. 3J) mice. (h) hypoglossal motor neurons; (v) visceral vagal motor

neurons. Scale bar shown in C = 50μm (Figs. 3A-3D) or 70μm (Figs. 3E-3J).

Figures 4A-4L **A switch in ventral interneuron fates in *Nkx6.1* mutant mice.**

Chx10 expression in V2 neurons at rostral cervical levels of E10.5 wild-type (Fig. 4A) and *Nkx6.1* mutant (Fig. 4B) embryos. En1 expression by V1 neurons at rostral cervical levels of wild-type (Fig. 4C) and *Nkx6.1* mutant (Fig. 4D) embryos. *Pax2* expression in a set of interneurons that includes V1 neurons ((Burrill et al. 1997) at caudal hindbrain levels of wild-type (Fig. 4E) and *Nkx6.1* mutant (Fig. 4F) embryos. (Figs. 4G and 4H) *Sim1* expression by V3 neurons in the cervical spinal cord of wild-type (Fig. 4G) and *Nkx6.1* mutant (Fig. 4H) embryos. *Evx1* expression by V0 neurons at caudal hindbrain levels of wild-type (Fig. 4I) and *Nkx6.1* mutant (Fig. 4J) embryos. *En1* (red) and *Lhx3* (green) expression by separate cell populations in the ventral spinal cord of E11 wild-type (Fig. 4K) and *Nkx6.1* mutant (Fig. 4L) embryos. Scale bar shown in B = 60μm (Figs. 4A-4D); 75μm (Figs. 4E, 4F); 70μm (Figs. 4G, 4J, 4H, 4J), 35μm (Figs. 4K and 4L).

Figure 5A-5B **Changes in progenitor domain identity and neuronal fate in the spinal cord of *Nkx6.1***

mutant embryos.

(Fig. 5A). In wild-type mouse embryos, cells in the *Nkx6.1* progenitor domain give rise to three classes of ventral neurons: V2 neurons, motor neurons (MN) and V3 neurons. V3 neurons derive from cells in the ventral most region of *Nkx6.1* expression that also express *Nkx2.2* and *Nkx2.9*. V1 neurons derive from progenitor cells that express *Dbx2* but not *Nkx6.1*. (Fig. 5B). In *Nkx6.1* mutant embryos the domain of Dbx2 expression by progenitor cells expands ventrally, and by embyonic day 12 [E12] occupies the entire dorsoventral extent of the ventral neural tube, excluding the floor plate. Checked area indicates the gradual onset of ventral *Dbx2* expression. This ventral shift in *Dbx2* expression is associated with a marked decrease in the generation of V2 neurons and motor neurons and a ventral expansion in the domain of generation of V1 neurons. A virtually complete loss of MN and V2 neurons is observed at cervical levels of the spinal cord. The generation of V3 neurons (and cranial visceral motor neurons at hindbrain levels) is unaffected by the loss of *Nkx6.1* or by the ectopic expression of *Dbx2*.

Sub a2

Figure 6 **Human Homeobox Protein Nkx6.1**. NCBI Accession No. P78426. (Inoue, H. et al., "Isolation, characterization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Amino acid sequence of human homeobox protein Nkx6.1.

Sub a3

Figure 7 **Human NK Homeobox Protein (Nkx6.1) gene, exon 1.** NCBI Accession No. U66797. Segment 1 of 3 (Inoue, H. et al., "Isolation, characterization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-682.

Sub a4

Figure 8 **Human NK Homeobox Protein (Nkx6.1) gene, exon 2.** NCBI Accession No. U66798. Segment 2 of 3 (Inoue, H. et al., "Isolation, characterization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic islet homeobox gene" Genomics 40(2):367-370, 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-185.

Sub a5

Figure 9 **Human NK Homeobox Protein (Nkx6.1) gene, exon 3 and complete cds.** NCBI Accession No. U66799. Segment 3 of 3 (Inoue, H. et al., "Isolation, character-ization, and chromosomal mapping of the human Nkx6.1 gene (NKX6a), a new pancreatic

islet homeobox gene" Genomics 40(2):367-370, 1997). Nucleic acid sequence encoding human homeobox protein Nkx6.1, bases 1-273. Protein encoded is shown in Fig. 7.

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DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method of converting a stem cell into a ventral neuron which comprises introducing into the stem cell a nucleic acid which expresses homeodomain transcription factor Nkx6.1 protein in the stem cell so as to thereby convert the stem cell into the ventral neuron.

In an embodiment of the above-described method of of converting a stem cell into a ventral neuron, the nucleic acid introduced into the stem cell incorporates into the chromosomal DNA of the stem cell. In a further embodiment of the method, the nucleic acid is introduced by transfection or transduction. In another further embodiment of the method, the ventral neuron is a motor neuron, a V2 neuron or a V3 neuron.

This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises: a) obtaining a nucleic acid sample from the subject; b) sequencing the nucleic acid sample; and c) comparing the nucleic acid sequence of step (b) with a Nkx6.1 nucleic acid sequence from a subject without motor neuron degenerative disease, wherein a difference in the nucleic acid sequence of step (b) from the Nkx6.1 nucleic acid sequence from the subject without motor neuron degenerative disease indicates that the subject has the motor neuron degenerative disease.

In an embodiment of the above-described method of

diagnosing a motor neuron degenerative disease in a subject the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.

This invention provides a method of diagnosing a motor neuron degenerative disease in a subject which comprises: a) obtaining a nucleic acid sample from the subject; b) performing a restriction digest of the nucleic acid sample with a panel of restriction enzymes; c) separating the resulting nucleic acid fragments by size fractionation; d) hybridizing the resulting separated nucleic acid fragments with a nucleic acid probe(s) of at least 15 nucleotide capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a human Nkx6.1 protein, wherein the sequence of the nucleic acid probe is labeled with a detectable marker, and hybridization of the nucleic acid probe(s) with the separated nucleic acid fragments results in labeled probe-fragment bands; e) detecting labeled probe-fragment bands, wherein the labeled probe-fragment bands have a band pattern specific to the nucleic acid of the subject; and f) comparing the band pattern of the detected labeled probe-fragment bands of step (d) with a previously determined control sample, wherein the control sample has a unique band pattern specific to the nucleic acid of a subject having the motor neuron degenerative disease, wherein identity of the band pattern of the detected labeled probe-fragment bands of step (d) to the control sample indicates that the subject has the motor neuron degenerative disease.

In an embodiment of the above-described method of diagnosing a motor neuron degenerative disease in a subject the nucleic acid is DNA. In a further embodiment of the above-described method the nucleic acid is RNA. In another embodiment the size fractionation in step (c) is effected by a polyacrylamide or agarose gel. In another embodiment the detectable marker is radioactive isotope, enzyme, dye, biotin, a fluorescent label or a chemiluminescent label. In yet another embodiment the motor neuron degenerative disease is amyotrophic lateral sclerosis or spinal muscular atrophy.

This invention provides a method of treating neuronal degeneration in a subject which comprises implanting in diseased neural tissue of the subject a neural stem cell which comprises an isolated nucleic acid molecule which is capable of expressing homeodomain Nkx6.1 protein under conditions such that the stem cell is converted into a motor neuron after implantation, thereby treating neuronal degeneration in the subject.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Materials and Methods

Generation of Nkx6.1 null mutation

A null mutation in *Nkx6.1* was generated by using gene targeting in 129-strain ES cells by excising an 800-bp NotI fragment containing part of exon 1 and replacing it by a PGK-neo cassette (Sander and German, unpubl.) Mutants were born at Mendelian frequency and died soon after birth; they exhibited movements only upon tactile stimulation.

Immunocytochemistry and in situ hybridization

Localization of mRNA was performed by in situ hybridization following the method of Schaeren-Wiemers and Gerfin-Moser (1993). The *Dbx2* riboprobe comprised the 5' EcoR1 fragment of the mouse cDNA (Pierani et al. 1999). Probes for other cDNAs were cited in the text and used as described therein. Protein expression was localized by indirect fluorescence immunocytochemistry or peroxidase immunocytochemistry (Briscoe et al. 1999; Ericson et al. 1997). *Nkx6.1* was detected with a rabbit antiserum (Briscoe et al. 1999). Antisera against Shh, Pax7, Isl1/2, HB9, Lhx3, Chx10, Phox2a/b, En1, and Pax2 have been described (Briscoe et al. 1999; Ericson et al. 1997). Fluorescence detection was carried out using an MRC 1024 Confocal Microscope (BioRad).

RESULTS AND DISCUSSION

To define the role of *Nkx6.1* in neural development, we compared patterns of neurogenesis in the embryonic spinal cord and hindbrain of wild-type mice and mice lacking *Nkx6.1* (Sander et al. 1998). In wild-type embryos, neural expression of *Nkx6.1* is first detected at spinal cord and caudal hindbrain levels at about embryonic day 8.5 (E8.5; Qiu et al. 1998; data not shown), and by E9.5 the gene is expressed throughout the ventral third of the neural tube (Figure 1A). The expression of *Nkx6.1* persists until at least E12.5 (Figures 1B, 1C; data not shown). *Nkx6.1* expression was also detected in mesodermal cells flanking the ventral spinal cord (Figures 1B, 1C). To define more precisely the domain of expression of Nkx6.1, we compared its expressions with that of ten homeobox genes - *Pax3, Pax7, Gsh1, Gsh2, Irx3, Pax6, Dbx1, Dbx1, Dbx2 and Nkx2.9* - that have been shown to define discrete progenitor cell domains along the dorsoventral axis of the ventral neural tube (Goulding et al. 1991; Valerius et al. 1995; Ericson et al. 1997; Pierani et al. 1999; Briscoe et al. 2000).

This analysis revealed that the dorsal boundary of *Nkx6.1* expression is positioned ventral to the boundaries of four genes expressed by dorsal progenitor cells: *Pax3*, *Pax7*, *Gsh1* and *Gsh2* (Figures 1I, 1N; and data not shown). Within the ventral neural tube, the dorsal boundary of *Nkx6.1* expression is positioned ventral to the domain of *Dbx1* expression and close to the ventral boundary of *Dbx2* expression (Figures 1G, 1H, and 1P). The domain of *Pax6* expression extends ventrally into the domain of *Nkx6.1*

expression (Figure 1O), whereas the expression of *Nkx2.2* and *Nkx2.9* overlaps with the ventral-most domain of *Nkx6.1* expression (Figures 1O, 1Q).

To address the function of *Nkx6.1* in neural development, we analyzed progenitor cell identity and the pattern of neuronal differentiation in *Nkx6.1* null mutant mice (Sander et al. 1998). We detected a striking change in the profile of expression of three homeobox genes, *Dbx2*, *Gsh1* and *Gsh2*, in *Nkx6.1* mutants. The domains of expression of *Dbx2*, *Gsh1* and *Gsh2* each expanded into the ventral neural tube (Figures 1K-1M; data not shown). At E10.5, *Dbx2* was expressed at high levels by progenitor cells adjacent to the floor plate, but at this stage ectopic *Dbx2* expression was detected only at low levels in regions of the neural tube that generate motor neurons (Figure 1K). By E12.5, however, the ectopic ventral expression of *Dbx2* had become more uniform, and now clearly included the region of motor neuron and V2 neuron generation (Figure 1L). Similarly, in *Nkx6.1* mutants, both *Gsh1* and *Gsh2* were ectopically expressed in a ventral domain of the neural tube, and also in adjacent paraxial mesodermal cells (Figure 1M; data not shown).

The ventral limit of Pax6 expression was unaltered in *Nkx6.1* mutants, although the most ventrally located cells within this progenitor domain expressed a higher level of Pax6 protein than those in wild-type embryos (Figures 1O, 1S). We detected no change in the patterns of expression of *Pax3*, *Pax7*, *Dbx1*, *Irx3*, *Nkx2.2*, or *Nkx2.9* in *Nkx6.1* mutant embryos (Figures 1R-1U; data not shown).

Importantly, the level of Shh expression by floor plate cells was unaltered in *Nkx6.1* mutants (Figures 1N and 1R). Thus, the loss of *Nkx6.1* function deregulates the patterns of expression of a selected subset of homeobox genes in ventral progenitor cells, without an obvious effect on Shh levels (Figures 1D, 1E). The role of Shh in excluding *Dbx2* from the most ventral region of the neural tube (Pierani et al. 1999) appears therefore to be mediated through the induction of Nkx6.1 expression. Consistent with this view, ectopic expression of *Nkx6.1* represses *Dbx2* expression in chick neural tube (Briscoe et al. 2000). The detection of sites of ectopic *Gsh1/2* expression in the paraxial mesoderm as well as the ventral neural tube, both sites of *Nkx6.1* expression, suggests that *Nkx6.1* has a general role in restricting *Gsh1/2* expression. The signals that promote ventral *Gsh1/2* expression in *Nkx6.1* mutants remain unclear, but could involve factors other than Shh that are secreted by the notochord (Hebrok et al. 1998).

The domain of expression of *Nkx6.1* within the ventral neural tube of wild-type embryos encompasses the progenitors of three main neuronal classes: V2 interneurons, motor neurons and V3 interneurons (Goulding et al. 1991; Ericson et al. 1997; Qiu et al. 1998; Briscoe et a. 1999, 2000; Pierani et al. 1999; Figures 2A-2D). We examined whether the generation of any of these neuronal classes is impaired in *Nkx6.1* mutants, focusing first on the generation of motor neurons. In *Nkx6.1* mutant embryos there was a marked reduction in the number of spinal motor neurons, as assessed by expression

of the homeodomain proteins Lhx3, Isl1/2 and HB9 (Arber et al. 1999; Tsuchida et al. 1994; Figures 2E-2L), and by expression of the gene encoding the transmitter synthetic enzyme choline acetyltransferase (data not shown). In addition, few if any axons were observed to emerge from the ventral spinal cord (data not shown). The incidence of motor neuron loss, however, varied along the rostrocaudal axis of the spinal cord. Few if any motor neurons were detected at caudal cervical and upper thoracic levels of *Nkx6.1* mutants analyzed at E11-E12.5 (Figures 2M, 2N, 2Q, 2R), whereas motor neuron number was reduced only by 50%-75% at more caudal levels (Figures 2O, 2P, 2S, 2T; data not shown). At all axial levels, the initial reduction in motor neuron number persisted at both E12.5 and p0 (Figures 2M-2T; data not shown), indicating that the loss of *Nkx6.1* activity does not simply delay motor neuron generation. Moreover, we detected no increase in the incidence of TUNEL$^+$ cells in *Nkx6.1* mutants (data not shown), providing evidence that the depletion of motor neurons does not result solely from apoptotic death.

The persistence of some spinal motor neurons in *Nkx6.1* mutants raised the possibility that the generation of particular subclasses of motor neurons is selectively impaired. To address this issue, we monitored the expression of markers of distinct subtypes of motor neurons at both spinal and hindbrain levels of *Nkx6.1* mutant embryos. At spinal levels, the extent of the reduction in the generation of motor neurons that populate the median (MMC) and lateral (LMC) motor columns

was similar in *Nkx6.1* mutants, as assessed by the number of motor neurons that coexpressed Isl1/2 and Lhx3 (defining MMC neurons, Figures 3A, 3B) and by the expression of *Raldh2* (defining LMC neurons, Sockanathan and Jessell 1998; Arber et al. 1999; Figures 3C, 3D). In addition, the generation of autonomic visceral motor neurons was reduced to an extent similar to that of somatic motor neurons at thoracic levels of the spinal cord of E12.5 embryos (data not shown). Thus, the loss of *Nkx6.1* activity depletes the major subclasses of spinal motor neurons to a similar extent.

At hindbrain levels, *Nkx6.1* is expressed by the progenitors of both somatic and visceral motor neurons (Figures 3E, 3F; data not shown). We therefore examined whether the loss of *Nkx6.1* might selectively affect subsets of cranial motor neurons. We detected a virtually complete loss in the generation of hypoglossal and abducens somatic motor neurons in *Nkx6.1* mutants, as assessed by the absence of dorsally generated HB9^{+} motor neurons (Figures 3G, 3H; data not shown, Arber et al. 1999; Briscoe et al. 1999). In contrast, there was no change in the initial generation of any of the cranial visceral motor neuron populations, assessed by coexpression of *Isl1* and *Phox2a* (Briscoe et al. 1999; Pattyn et al. 1997) within ventrally generated motor neurons (Figures 3I, 3J; data not shown). Moroever, at rostral cervical levels, the generation of spinal accessory motor neurons (Ericson et al. 1997) was also preserved in *Nkx6.1* mutants (data not shown). Thus, in the hindbrain the loss of *Nkx6.1* activity selectively

eliminates the generation of somatic motor neurons, while leaving visceral motor neurons intact. Cranial visceral motor neurons, unlike spinal visceral motor neurons, derive from progenitors that express the related *Nkx* genes *Nkx2.2* and *Nkx2.9* (Briscoe et al. 1999). The preservation of cranial visceral motor neurons in *Nkx6.1* mutant embryos may therefore reflect the dominant activities of *Nkx2.2* and *Nkx2.9* within these progenitor cells.

We next examined whether the generation of ventral interneurons is affected by the loss of *Nkx6.1* activity. V2 and V3 interneurons are defined, respectively, by expression of *Chx10* and *Sim1* (Arber et al. 1999; Briscoe et al. 1999; Figures 4A, 4G). A severe loss of Chx10 V2 neurons was detected in *Nkx6.1* mutants at spinal cord levels (Figure 4B), although at hindbrain levels of *Nkx6.1* mutants ~50% of V2 neurons persisted (data not shown). In contrast, there was no change in the generation of *Sim1* V3 interneurons at any axial level of *Nkx6.1* mutants (Figure 4H). Thus, the elimination of *Nkx6.1* activity affects the generation of only one of the two major classes of ventral interneurons that derive from the Nkx6.1 progenitor cell domain.

Evx1$^+$, Pax2$^+$ V1 interneurons derive from progenitor cells located dorsal to the *Nkx6.1* progenitor domain, (Figure 4B) within a domain that expresses *Dbx2*, but not *Dbx1* (Burrill et al. 1997; Matise and Joyner 1997; Pierani et al. 1999). Because *Dbx2* expression undergoes a marked ventral expansion in *Nkx6.1* mutants, we examined whether

there might be a corresponding expansion in the domain of generation of V1 neurons. In *Nkx6.1* mutants, the region that normally gives rise to V2 neurons and motor neurons now also generated V1 neurons, as assessed by the ventral shift in expression of the En1 and Pax2 homeodomain proteins (Figures 4B, 4C, 4E, 4F). Consistent with this, there was a two- to threefold increase in the total number of V1 neurons generated in *Nkx6.1* mutants (Figures 4C, 4D). In contrast, the domain of generation of Evx1/2 V0 neurons, which derive from the *Dbx1* progenitor domain (Pierani et al. 1999), was unchanged in *Nkx6.1* mutants (Figures 4I, 4J). Thus, the ventral expansion in *Dbx2* expression is accompanied by a selective switch in interneuronal fates, from V2 neurons to V1 neurons. In addition, we observed that some neurons within the ventral spinal cord of *Nkx6.1* mutants coexpressed the V1 marker *En1* and the V2 marker *Lhx3* (Figures 4K, 4L). The coexpression of these markers is rarely if ever observed in single neurons in wild type embryos (Ericson et al. 1996). Thus, within individual neurons in *Nkx6.1* mutants, the ectopic program of V1 neurogenesis appears to be initiated in parallel with a residual, albeit transient, program of V2 neuron generation. This result complements observations in *Hb9* mutant mice, in which the programs of V2 neuron and motor neuron generation coincide transiently within individual neurons (Arber et al. 1999; Thaler et al. 1999).

Taken together, the findings herein reveal an essential role for the *Nkx6.1* homeobox gene in the specification of regional pattern and neuronal fate in the ventral half of

the mammalian CNS. Within the broad ventral domain within which *Nkx6.1* is expressed (Figure 5A), its activity is required to promote motor neuron and V2 interneuron generation and to restrict the generation of V1 interneurons (Figure 5B). It is likely that the loss of motor neurons and V2 neurons is a direct consequence of the loss of *Nkx6.1* activity, as the depletion of these two neuronal subtypes is evident at stages when only low levels of *Dbx2* are expressed ectopically in most regions of the ventral neural tube. Nonetheless, it can not be excluded that low levels of ectopic ventral Dbx2 expression could contribute to the block in motor neuron generation. Consistent with this view, the ectopic expression of *Nkx6.1* is able to induce both motor neurons and V2 neurons in chick neural tube (Briscoe et al. 2000). V3 interneurons and cranial visceral motor neurons derive from a set of *Nkx6.1* progenitors that also express *Nkx2.2* and *Nkx2.9* (Briscoe et al. 1999, Figure 5A). The generation of these two neuronal subtypes is unaffected by the loss of *Nkx6.1* activity, suggesting that the actions of *Nkx2.2* and *Nkx2.9* dominate over that of Nkx6.1 within these progenitors. The persistence of some spinal motor neurons and V2 neurons in *Nkx6.1* mutants could reflect the existence of a functional homologue within the caudal neural tube.

The role of *Nkx6.1* revealed in these studies, taken together with previous findings, suggests a model in which the spatially restricted expression of *Nkx* genes within the ventral neural tube (Figure 5) has a pivotal role in defining the identity of ventral cell types

induced in response to graded Shh signaling. Strikingly, in *Drosophila*, the *Nkx* gene *NK2* has been shown to have an equivalent role in specifying neuronal fates in the ventral nerve cord (Chu et al. 1998; McDonald et al. 1998). Moreover, the ability of *Nkx6.1* to function as a repressor of the dorsally expressed *Gsh1/2* homeobox genes parallels the ability of *Drosophila NK2* to repress *Ind*, *a Gsh1/2*-like homeobox gene (Weiss et al. 1998). Thus, the evolutionary origin of regional pattern along the dorsoventral axis of the central nervous system may predate the divergence of invertebrate and vertebrate organisms.

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